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HEMOPHILIC BLOOD OF MAN

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NOTE UPON THE PRESENCE OF HEPARIN IN NORMAL AND HEMOPHILIC BLOOD OF MAN

W. H. HOWELL

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In a recent paper the author (1925) has given evidence to show that the delayed clotting or incoagulability of blood of a peptonized dog is caused by the presence of an excess of heparin in the blood. I have shown, moreover, that in normal dog's blood a detectable amount of heparin is present. The method used to demonstrate these points was briefly as follows: The blood received from an artery was oxalated and centrifugalized. In some cases the peptonized blood was centrifugalized without oxalating. The clear plasma was pipetted off and precipitated by the addition of an equal volume of acetone. The precipitate was collected by centrifugalization and was boiled for two hours in a large volume of 95 per cent methyl alcohol, using a reflux condenser. The solution was filtered, washed with hot methyl alcohol and the precipitate was dried and powdered. This material was then extracted for 15 to 30 minutes with a solution of 1 per cent sodium chloride, the extract was filtered and the filtrate was precipitated by an equal volume of acetone. This precipitate was collected by centrifugalizing and dried. It was then extracted with a little water, filtered and the filtrate evaporated to dryness over the water-bath. The residue thus obtained was used for testing for the presence of heparin.

More recently this method has been varied with advantage in the following way: The whole blood has been used in place of the centrifugalized plasma. In the case of human blood the procedure has been to insert a good-sized hypodermic needle into an arm vein and allow the blood to flow through an oiled tube directly into acetone. Usually 100 cc. of blood were taken from the vein and received into 100 cc. of acetone, the mixture being stirred gently during the flow. The precipitate was separated by centrifugalizing and was boiled for two hours with an excess of methyl alcohol. The solution was filtered, the precipitate was washed with hot methyl alcohol, dried and powdered. The dried material was then extracted for a few minutes with a boiling solution (150 cc.) of 1 per cent sodium chloride. The filtered extract was precipitated by acetone and treated as described above.

With the small amount of material thus obtained tests were made for the presence of heparin in human blood as follows. The entire material

was extracted with 2 cc. of a 1 per cent solution of sodium chloride. The extract was filtered and the filtrate was compared with an equal volume of 1 per cent sodium chloride solution in regard to its property of delaying the coagulation of freshly drawn blood and of causing an increase in anti-thrombin when added to oxalated plasma.

Anticoagulant action. The anticoagulant action was tested by mixing 0.5 cc. or 1 cc. of the filtrate with 1 cc. of normal human blood taken from an arm vein by means of a syringe:

Experiment V. Control: 0.5 cc. salt solution plus 1 cc. blood = clot in 7 to 8 minutes.

Extract: 0.5 cc. extract plus 1 cc. blood = clot, imperfect, in 40 to 50 minutes.

Experiment VI. Control: 1 cc. salt solution plus 1 cc. blood = clot in 12 minutes.

Extract: 1 cc. extract plus 1 cc. blood = clot in 50 minutes.

Experiment VII. Control: 1 cc. salt solution plus 1 cc. blood = clot in 10 minutes.

Extract: 1 cc. extract plus 1 cc. blood = clot in 48 minutes.

Antithrombin effect. It is one of the characteristics of heparin that when added to serum or plasma it causes an increase in the content of anti-thrombin as tested upon definite mixtures of fibrinogen and thrombin. In these experiments oxalated cat's plasma was used after previously heating to 56°C. to destroy the fibrinogen. The thrombin was made from a dried specimen prepared by my method and now some 12 years old. Ten milligrams of the thrombin were dissolved in 15 cc. of 1 per cent solution of sodium chloride. The fibrinogen solutions were made from oxalated cat's plasma by the ammonium sulphate method. They did not clot spontaneously nor upon the addition of the calcium chloride and were, therefore, free from thrombin and prothrombin.

When the thrombin solution was added to the fibrinogen solution clotting occurred in a few minutes. The time varied in different specimens of fibrinogen, but was always short, for example:

Thrombin 4 drops + Fibrinogen 8 drops = clot in 2 minutes.

Thrombin 3 drops + Fibrinogen 8 drops = clot in 2 to 3 minutes

Thrombin 2 drops + Fibrinogen 8 drops = clot in 4 to 5 minutes

Thrombin 1 drop + Fibrinogen 8 drops = clot in 8 to 9 minutes

In the test for antithrombin in the heated oxalated plasma two solutions were prepared—one, a control, consisted of equal parts of the heated oxalated plasma and a 1 per cent solution of sodium chloride, the other, of equal parts of the heated oxalated plasma and the extract prepared from the normal human blood as described above.

Experiment VII. Antithrombin in the control mixture:

Thrombin 1 drop + solution 1 drop, incubation 15 minutes, fibrinogen 8 drops = clot in 5 hours

Thrombin 2 drops + solution 1 drop, incubation 15 minutes, fibrinogen 8 drops = clot in 25 minutes

Thrombin 3 drops + solution 1 drop, incubation 15 minutes, fibrinogen 8 drops = clot in 15 minutes

Thrombin 4 drops + solution 1 drop, incubation 15 minutes, fibrinogen 8 drops = clot in 10 minutes

Antithrombin in the blood extract mixtures:

Thrombin 1 drop + solution 1 drop, incubation 15 minutes, fibrinogen 8 drops = clot between 5 and 24 hours

Thrombin 2 drops + solution 1 drop, incubation 15 minutes, fibrinogen 8 drops = clot between 110 and 130 minutes

Thrombin 3 drops + solution 1 drop, incubation 15 minutes, fibrinogen 8 drops = clot in 25 minutes

Thrombin 4 drops + solution 1 drop, incubation 15 minutes, fibrinogen 8 drops = clot in 20 minutes

The antithrombin content of the heated oxalated plasma was, therefore, increased by the addition of the extract prepared from normal blood. That this extract did not itself contain any antithrombin was demonstrated by a control experiment, as follows:

Thrombin 1 drop + blood extract 1 drop, incubation of 15 minutes + fibrinogen 8 drops = clot in 7 to 8 minutes

Thrombin 2 drops + blood extract 1 drop, incubation of 15 minutes + fibrinogen 8 drops = clot in 3 to 4 minutes

Thrombin 3 drops + blood extract 1 drop, incubation of 15 minutes + fibrinogen 8 drops = clot in 3 to 4 minutes

Control:

Thrombin 1 drop + saline 1 drop, incubation of 15 minutes + fibrinogen 8 drops = clot in 6 to 7 minutes

Thrombin 2 drops + saline 1 drop, incubation of 15 minutes + fibrinogen 8 drops = clot in 3 to 4 minutes

Thrombin 3 drops + saline 1 drop, incubation of 15 minutes + fibrinogen 8 drops = clot in 3 to 4 minutes

It seems certain from these results that normal human blood contains an anticoagulant different from antithrombin. The method by which it was obtained and its power to increase the antithrombin content of serum or plasma serve to identify this anticoagulant with heparin.

HEMOPHILIC BLOOD. The fact that the heparin in the incoagulable blood of the peptonized dog, as tested by the above methods, is greatly increased suggested the possibility that the delayed clotting of hemophilic blood may be due to the same cause. Weil (1906) was the first to suggest that the delayed clotting of hemophilic blood is due to the presence of an excess of some anticoagulant. In two out of three cases he found that the serum of hemophilic blood delayed the clotting of normal blood. Other authors have not been able to confirm this result. Addis (1911) obtained entirely negative results. The addition of oxalated hemophilic plasma to oxalated normal plasma did not delay the clotting of the latter when recalcified. Howell (1914) examined the blood of hemophiles for the presence of antithrombin, by his method, as compared with normal bloods. While, in general, an excess was found the difference was slight

and in some cases was lacking. A similar result was obtained by Minot and Lee (1916).

As further proof of the absence of an excess of anticoagulant, Feissly (1923) states that thrombin added to hemophilic plasma causes it to clot as readily as it does normal citrated plasma. This argument applies, however, only to an anticoagulant of the nature of antithrombin, and not to heparin whose effect is not exerted upon thrombin but upon the antecedent substance, the prothrombin. In another paper Feissly (1925a) states that when prothrombin (proserozyme) is prepared from hemophilic plasma by the Bordet method of adsorption upon tricalcium phosphate, this precipitate, when redissolved in saline, possesses the property of preventing the coagulation of a recalcified citrated plasma, and in a general discussion of the subject he considers the possibility of the existence of some stabilizing or inhibitory factor in hemophilic blood (1925b).

Davidson and McQuarrie (1925) state that a suspension of platelets prepared from hemophilic blood when added to normal oxalated plasma causes a marked prolongation of the coagulation time upon recalcification, whereas a suspension of normal platelets has no such effect. This observation, however, is contradicted by the similar experiments of Feissly and Fried (1924). They found that suspensions of platelets from hemophilic plasmas had the same effect as similar suspensions from normal plasmas in hastening the coagulation of a recalcified oxalated plasma.

Minot and Lee (1916) obtained the same result when the optimum amount of calcium was used for recalcification.

There is obviously some disagreement among workers in regard to the existence in hemophilic blood of an anticoagulant material in excess of that present in normal blood. So far as antithrombin is concerned, the direct evidence quoted above indicates that it is not present in amounts distinctly above normal; but since I have shown that normal blood contains another anticoagulant, heparin, which is an antiprothrombin, it seemed desirable to study hemophilic blood with reference to its content in this latter substance, using the methods described above. Two young men, J. Y. and F. Y., belonging to the hemophilic family that I studied in 1914, were used in these experiments. These individuals had been kept under observation during the intervening period. From time to time they have had trouble from spontaneous hemorrhages in the joints or kidneys and have been treated by transfusion. One of them, J. Y., at the time of these experiments had been undergoing treatment for six months consisting of periodic injections intravenously of calcium chloride. While the result of this treatment seemed to be beneficial and the coagulation time of his blood was reduced somewhat, his general condition was not entirely satisfactory. When examined in 1913 the coagulation time of J. Y.'s blood was from 3 to 4 hours, examined again in 1924 it was 2 hours. After the above treatment had been continued for six months the coagulation time

was from 1½ to 2 hours. The coagulation time of the blood of F. Y. taken in February and March, 1924, was from 1 hour, 15 minutes to 1 hour, 40 minutes. At the time that these experiments were made, May and June, 1925, the coagulation time was much longer, approximately 4 hours. During the following summer this case was taken into the hospital in consequence of serious hemorrhages from the kidney and the knee-joint and was given several transfusions. It should be stated that the coagulation time was determined in all cases by taking a specimen from the arm vein with a syringe and emptying 2 cc. into a clean tube, having a diameter of 15 mm.

Blood (100 cc.) was taken from the arm vein of these two hemophilics, by means of a syringe or through a needle and tube. In some cases it was oxalated and centrifugalized to get the plasma. In other cases it was run directly into an equal volume of acetone. The acetone precipitate was treated as in the case of the normal blood to obtain the heparin, and the final material was examined for its anticoagulant action and its property of increasing the antithrombin content of heated plasma in comparison with an equal specimen of normal blood treated by the same method.

The results were not consistent. In some cases the hemophilic blood contained a greater amount of the heparin than the normal blood judged by the two tests described, while in other cases it contained practically the same amount or even less. The hemophilic blood seemed to vary somewhat in its content of heparin and as between the two hemophilic cases examined the blood of F. Y. contained uniformly more than that of J. Y. Without giving the full details of the experiments, the general results were as follows:

Anticoagulant action. The anticoagulant action was tested by mixing 1 cc. of the extract with 1 cc. of freshly drawn blood. In the control 1 cc. of a 1 per cent solution of sodium chloride was used in place of the extract.

	CONTROL	HEPARIN EXTRACT FROM NORMAL HUMAN BLOOD	HEPARIN EXTRACT FROM HEMOPHILIC BLOOD
	<i>minutes</i>	<i>minutes</i>	<i>minutes</i>
Experiment I. Using cat's blood for coagulation.....	28	45	50 (J. Y.)
Experiment II. Using cat's blood for coagulation.....	17	40	100 (J. Y.)
Experiment III. Using human blood for coagulation.....	27	27	45 (J. Y.)
Experiment IV. Using human blood for coagulation.....	10	23	28 (J. Y.) 35 (F. Y.)
Experiment VI. Using human blood for coagulation.....	12	50	40 (F. Y.)
Experiment VII. Using human blood for coagulation.....	10	48	28 (J. Y.) 80 (F. Y.)

Antithrombin action. Experiment I. Control:

Thrombin 1 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 20 minutes

Thrombin 2 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 15 minutes

Thrombin 3 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 15 minutes

Thrombin 4 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 10 minutes

Extract of normal blood:

Thrombin 1 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 25 minutes

Thrombin 2 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 15 minutes

Thrombin 3 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 15 minutes

Thrombin 4 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 10 minutes

Extract of hemophilic blood:

Thrombin 1 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 25 minutes

Thrombin 2 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 15 minutes

Thrombin 3 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 15 minutes

Thrombin 4 + extract 1—incubation 15 minutes + fibrinogen 8 = clot in 10 minutes

Experiment 2. Following the same procedure the clotting times were:

Control:

Thrombin 1 drop = clot in 50 minutes

Thrombin 2 drops = clot in 20 minutes

Extract of normal blood:

Thrombin 1 drop = clot in 60 minutes

Thrombin 2 drops = clot in 20 minutes

Extract of hemophilic blood:

Thrombin 1 drop = clot in 75 minutes

Thrombin 2 drops = clot in 25 minutes

Experiment IV. Control:

Thrombin 1 drop = clot in 8 to 9 hours

Thrombin 2 drops = clot in 120 minutes

Thrombin 3 drops = clot in 60 minutes

Thrombin 4 drops = clot in 25 minutes

Extract of normal blood:

Thrombin 1 drop = clot in 8 to 9 hours

Thrombin 2 drops = clot in 120 minutes

Thrombin 3 drops = clot in 60 minutes

Thrombin 4 drops = clot in 25 minutes

Extract of hemophilic blood:

Thrombin 1 drop = clot (beginning) after 14 hours

Thrombin 2 drops = clot in 165 to 190 minutes

Thrombin 3 drops = clot in 95 minutes

Thrombin 4 drops = clot in 55 minutes

Experiment VI. Control:

Thrombin 1 drop = clot after 18 hours
Thrombin 2 drops = clot after 18 hours
Thrombin 3 drops = clot after 18 hours
Thrombin 4 drops = clot after 105 minutes

Extract of normal blood:

Thrombin 1 drop = clot after 18 hours
Thrombin 2 drops = clot after 18 hours
Thrombin 3 drops = clot after 18 hours
Thrombin 4 drops = clot in 160 minutes

Extract of hemophilic blood:

Thrombin 1 drop = clot after 18 hours
Thrombin 2 drops = clot after 18 hours
Thrombin 3 drops = clot in 160 minutes
Thrombin 4 drops = clot in 55 minutes

Experiment VII. Control:

Thrombin 1 drop = clot between 5 and 7 hours
Thrombin 2 drops = clot in 25 minutes
Thrombin 3 drops = clot in 15 minutes
Thrombin 4 drops = clot in 10 minutes

Extract of normal blood:

Thrombin 1 drop = clot between 5 and 24 hours
Thrombin 2 drops = clot in 110 to 130 minutes
Thrombin 3 drops = clot in 25 minutes
Thrombin 4 drops = clot in 20 minutes

Extract of hemophilic blood:

Thrombin 1 drop = no clot after 24 hours
Thrombin 2 drops = clot between 5 and 24 hours
Thrombin 3 drops = clot in 40 minutes
Thrombin 4 drops = clot in 20 minutes

CONCLUSION

The results from both of these tests show that the hemophilic blood like the normal blood contains heparin in amounts that vary within certain limits, but there is no clear indication of a greater quantity of heparin in the hemophilic blood. While in some cases the heparin was greater than in the control of normal blood, the difference was not decisive, and in other cases there was no difference or the relation was reversed. The method used to extract the heparin was not quantitative, and might have been subject to unknown variables that confused the results. Still when the two specimens were put through exactly the same process, any definite excess of heparin in the hemophilic blood, if present, ought to have been made as clearly evident as in the case of the blood of the peptonized dog. The results, therefore, indicate that the hemophilic blood contains no excess of heparin beyond the normal limits, and the delayed coagulation can not be explained on this ground.

BIBLIOGRAPHY

- ADDIS. 1911. Journ. Path. and Bact., xv, 427.
- DAVIDSON AND McQUARRIE. 1925. Johns Hopkins Hosp. Bull., May.
- FEISSLY. 1923. Bull. et Mém. d. l. Soc. Méd. d. Hôp. d. Paris, December 14.
- 1925a. Compt. rend. d. Seances d. l. Soc. d. Biol., November 25.
- 1925b. Jahrb. f. Kinderheilk., cx, 297.
- FEISSLY AND FRIED. 1924. Klin. Wochenschr., May 6.
- HOWELL. 1914. Arch. Int. Med., January.
1925. This Journal, lxxi, 553.
- MINOT AND LEE. 1916. Arch. Int. Med., October.
- WEIL. 1906. Bull. et Mém. d. l. Soc. Méd. d. Hôp. d. Paris, October 26.

